Effects of Chemical Composition of Anthocyanin-rich Commodities on Their Chemoprotective Properties

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ABSTRACT

Anthocyanins are potent antioxidants and may be chemoprotective. However, the structure/function relationships are not well understood. Our objective was to determine the effect of chemical structure on the chemoprotective effects of anthocyanins on colon cancer as well as to determine the interaction with other phenols present. Anthocyanin-rich extracts (AREs) with different anthocyanin profiles from purple corn (*Zea mays* L.), chokeberry (*Aronia meloncarpa* E.), bilberry (*Vaccinium myrtillus* L.), purple carrot (*Daucus carota* L.), grape (*Vitis vinifera*), radish (*Raphanus sativus*), and elderberry (*Sambucus nigra* L.) were tested to determine the concentration needed to inhibit growth of colon cancer cells (HT29) by 50% (GI50). An anthocyanin fraction (ACN) and other phenolics fraction (OPF) were separated with a C18-cartridge and tested for synergistic, additive or antagonistic effect. All AREs inhibited colon cancer cell proliferation to varying degrees. Purple corn ARE showed the highest growth inhibition (GI50~14μg/ml), followed by chokeberry and bilberry. Radish showed the lowest inhibition (GI50~131μg/ml). This may be attributed to the presence of anthocyanin diglycosides, type of aglycone (pelargonidin) and/or cinnamic acid acylations. ACN, rather than other OPF, played an important role on the chemoprotective effects of AREs although both were mostly additive interaction to the total inhibitory effects. Saponification of purple corn ACN resulted on an increased inhibitory effect of HT29 cell proliferation, suggesting that non-acylated anthocyanins are more effective chemoprotective agents than their acylated counterpart. Conclusions are that anthocyanin-based colorants may be chemoprotective and therefore valuable ingredients for functional foods. Anthocyanin-rich commodities graded according to their chemoprotection will provide information for further application in function foods and crop and cultivar selection. Our results should provide light on what anthocyanin structures to choose for increased chemoprotection

INTRODUCTION

Anthocyanins are a class of flavonoid compounds responsible for the bright attractive red, orange, purple, and blue colors of most fruits and vegetables. Interest in anthocyanins has increased due to their color characteristics as certification-exempt colorants and health benefits as value-added ingredients.[1] Anthocyanins are the most abundant flavonoids in the diet with a daily consumption of about 200 mg/person,[2] much higher than the average intake of other flavonoids which were 23 mg/person totally.[3] Anthocyanins are rich in many foods: berries, purple carrot, purple corn, red radish, red cabbage, and other red, purple or blue plant foods. They may contribute to cancer preventive potentials of fruits and vegetables which are associated with the decreased cancer risk from epidemiological studies.[4]

Colon cancer is the 3rd most common cancer and the 3rd leading cause of cancer death for both men and women in the United States.[5] Anthocyanin-rich foods and anthocyanin pigments have been suggested as potential foods or food ingredients to reduce the risk of colon cancer from recent studies.[6-13,15] Anthocyanins have been consumed for many years without any apparent adverse effects and anthocyanin-rich extract from edible berries didn't have toxic effect to normal colon cells (NCM460) at the same concentration range which caused inhibitory effect on the growth of human cancer cells (HT29) in our previous study.[13]

There are six aglycones (anthocyanidins) more commonly found in nature. Since each aglycone may be glycosylated and acylated by different sugars, cinnamic and aliphatic acids and over 600 structurally distinct anthocyanins have been identified in nature.[14] However, the structure/function relationship of anthocyanins is still not clear. To understand the effects of anthocyanin structure on their biological activity is important for industries to choose anthocyanin-rich sources with increased health benefits and also meaningful for crop and cultivar selection.

ABBREVIATIONS

ARE, anthocyanin-rich extract; **ACN,** anthocyanin fraction; **OPF,** other phenol fraction; **AP,** reconstitution of anthocyanin fraction and other phenol fraction; **ACN_SA**, saponified anthocyanin-fraction; **GI_{50}**, the concentration of sample is required to inhibit 50% of cell growth; **GI tract:** gastrointestinal tract.

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OBJECTIVES

- To determine and compare the chemopreventative effects of anthocyanin-rich extracts from different sources on colon cancer cells
- To evaluate synergistic, additive or antagonistic effect between anthocyanins with other phenols
- To determine the effects of chemical structural modification on the chemopreventative activity of anthocyanins

MATERIALS & METHODS

HT29 cell line. A HT29 cell line derived from a colorectal adenocarcinoma was grown in McCoy's 5A medium, which was supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5 % CO2 atmosphere.

Sample preparation. Commercial AREs were semi-purified by C18 Sep-Pak solid cartridge.[16] When most of methanol was removed by rotary evaporation at 40 ° C and residue was added up to about 10 mL with deionized water and frozen for further lyophilization.

Anthocyanin fraction (ACN) and other-phenol fraction (OPF) were prepared by C18 Sep-Pak solid cartridge. When most of methanol in fractions was removed by rotary evaporation at 40 ° C, and each residue was added up to about 10 mL with deionized water and frozen for lyophilization.

Cell growth inhibition The HT29 cell was plated at 1.3 × 10^4 cells/well in 24-well plates in McCoy's 5A medium containing 10% FBS. Cells were allowed to grow 24 hours to attain log phase growth at the time of sample addition (time 0). HT29 cell growth was determined after additional 48 hr of incubation with different levels of anthocyanins by using the sulforhodamine B assay.

Sulforhodamine B assay. The detailed methodology for SRB assay was described by Skehan.[17] The absorbance at wavelength of 565 nm was measured.

Monomeric anthocyanins. The total monomeric anthocyanin content was measured by the pH differential method.[18]

Total phenols. Total phenols were measured using a modified method of microscale protocol for Folin-Ciocalteu colorimetry.[19]

Alkaline hydrolysis of anthocyanins. The anthocyanin fraction from purple corn extract (1mL) was saponified with 10 mL of 10% aqueous KOH.[20]

Statistical analysis. Regression curve and equation on the growth inhibition of cells at different ARE treatments were analyzed by SPSS (14.0) software in regression model. Tukey HSD test was conducted to evaluated mean differences among values of GI50 or growth inhibition (%) when at same concentration in one-way ANOVA model and values of $p<0.05$ were considered significant. Student T test also was used to evaluated the difference of mean and a fixed value at the level of $p<0.05$.

ANTHOCYANIN SOURCES

A. Anthocyanin-rich sources that contains only non-aycylated anthocyanins

Chokeberry (*Aronia meloncarpa* E.)

Bilberry (*Vaccinium myrtillus* L.)

Elderberry (*Sambucus nigra* L.)

B. Anthocyanin-rich sources that contains non-aycylated as well as acylated anthocyanins

Grape (*Vitis vinifera*)

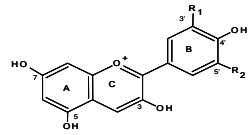
Purple corn (*Zea mays* L.)

Purple carrot (*Daucus carota* L.)

Radish (*Raphanus sativus*)

RESULTS

Structures of common anthocyanidins in fruits and vegetables

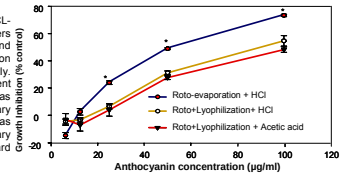


Basic structure of anthocyanidins

Anthocyanidin	Abbreviation	Substitute R_1	Substitute R_2	λmax (nm) visible spectra	Molecular Weight
Pelargonidin	Pg	H	H	494 (orange)	271
Cyanidin	Cy	OH	H	506 (orange-red)	287
Delphinidin	Dp	OH	OH	508 (red)	303
Peonidin	Pn	OCH_3	H	506 (orange-red)	301
Petunidin	Pt	OCH_3	OH	508 (red)	317
Malvidin	Mv	OCH_3	OCH_3	510 (bluish-red)	331

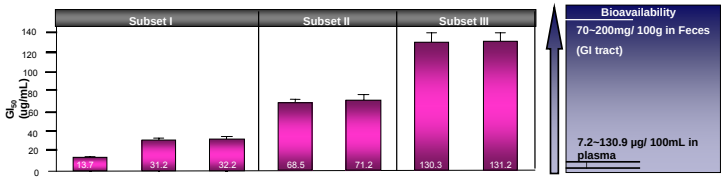
Methodology Development

Anthocyanin sample prepared in a manner with roto-evaporation and 0.01%-HCL-acidified solvents showed significantly higher inhibition of HT-29 cells than others ($p<0.05$) and the sample prepared with rotary evaporation plus lyophilization and 0.01%-HCl-acidifed solvents still showed higher inhibition than rotary evaporation plus lyophilization and 1%-acetic-acid-acidifed solvents although not significantly. Rotoevaporator + HCl: sample was prepared by 0.01%-HCl-acidified solvent which was removed by rotoevaporator; (Roto+Lyophilization) + HCl: sample was prepared by 0.01%-HCl-acidified solvent which was removed by rotary evaporation plus lyophilization; (Roto+Lyomphilization) + acetic acid: sample was preprared by 1%-acetic-acid-acified solvent which was removed by rotary evaporation plus lyophilization. Values are represented at mean ± standard error (n=4).



The chemoprotective activity of anthocyanin-rich extracts from seven natural sources

A. Comparison of GI_{50} values of anthocyanin-rich sources and comparison with anthocyanin bioavailability



B. Anthocyanin profiles in seven commercial anthocyanin-rich sources.

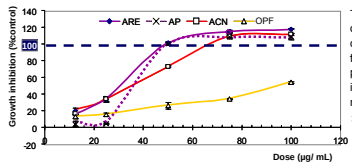
Source	Anthocyanidin	Glycosylation	Acylation
Purple corn (*Zea may* L.)	Cy, Pt, Pn	C3: mono-glycoside	One aliphatic acid
Chokeberry (*Aronia meloncarpa* E.)	Cy	C3: mono-glycoside	None
Bilberry (*Vaccinium myrtillus* L.)	Dp, Cy, Pt, Pn, Mv	C3:mono-glycoside	None
Purple carrot (*Daucus carota* L.)	Cy	**C3: di-, tri-glycoside**	**One cinnamic acid**
Grape (*Vitis inifera*)	Cy, Dp, Pt, Mv	C3: mono-glycoside	**One cinnamic acid**
Elderberry (*Sambucus nigra* L.)	Cy	**C3: mono-;di-glycoside C3 and C5: di-, tri-glycoside**	None
Radish (*Raphanus sativus* L.)	**Pg**	**C3 and C5: tri-glycoside**	**One or two cinnamic acids**

A. The GI50 of anthocyanin-rich extracts (based on the monomeric anthocyanins) from seven natural sources on the growth inhibition of HT-29 colon cell line. The natural sources were graded into different homogenous subsets of GI50 means of corresponding anthocyanin-rich extract (Tukey HSD, $p<0.05$). Anthocyanin-rich extracts were significantly different among groups while anthocyanins-rich extracts within same group were not significantly different. Values are represented as equivalents of cyanidin 3-glucoside (μg/ mL).

The GI50 values of these sources were much lower than the level of anthocyanins (70~200 mg/100g wet feces) in rat feces[21] suggesting that all AREs could be present in enough concentrations in the GI tract as to exert their chemprotective activity in vivo. These GI_{50} values was much lower than the reported concentrations of anthocyanins in plasma (0.1~1.8%) of anthocyanin consumption if consumption of anthocyanin is up to 200 mg/ person daily[2], suggesting that these anthocyanins could be less effective to breast, lung, and prostate cancers which need enough bioavailability of phytochemicals in blood stream.

B. GI_{50} values range from ~14 to ~131 μg/mL, about almost 10-time difference among these sources. This difference might be due to the presence of glycosylation, cinnamic acids acylated with sugar moieties, or to the type of aglycones. Radish, elderberry, and purple carrot are rich in anthocyanins with di- and tri-glycosides. Cinnamic acids may have an impact on the biological activity of anthocyanin aylated. Pelargonidin may exert lower activity on growth inhibition of HT-29 cells than other anthocyanidins.

Interaction between anthocyanins and other phenols in ARES



The cytostatic and cytotoxic effects of chokeberry ARE was observed on HT-29 cell. Anthocyanin fraction, rather than other phenols, played a major effect on ARE inhibitory effect. Values are represented as mean absorbance ± standard error (n=4).

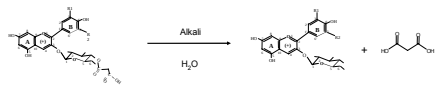
The AP reconstituted from anthocyanin and other phenol fractions according to their original ratio in chokeberry ARE, showed inhibitory effect very similar to the AREs.

Source	Combination Index at GI_{25}	GI_{50}	GI_{100}
Anthocyanins + Other Phenols	1.27	1.18	1.30*
Combination Effect	Additive	Additive	Antagonistic

Combination index (CI) values of the interaction between anthocyanin fraction and other phenols. CI<1, =1, and >1 indicate synergism, additive effect and antagonism, respectively. Each value represents the mean of three independent experiments carried out in 4 replicates. Student t-test were computed to evaluate if significant differences in the mean CI values comparing with a null hypothesized CI of 1 (* $p<0.05$). Values= Means ± Standard error (n=3).

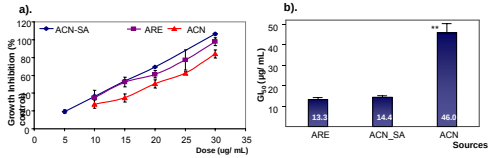
Effects of Chemical modification of anthocyanins on chemoprotection

A. Saponification reaction



Acylated anthocyanin — Non-acylated anthocyanin

B. Comparison of chemoprotective activity of anthocyanins in Purple corn after and before saponification



a): The saponified anthocyanins showed higher inhibitory effect in dose-depend manner than acylated counterpart and comparable to ARE from purple corn. **b):**. The GI_{50} of anthocyanin-rich extract and saponified anthocyanin-fraction were significantly lower than the one of anthocyanin fraction from purple corn on the growth inhibition of HT-29 colon cell line ($p<0.01$), suggesting non-acylated anthocyanin exerted better inhibitory effect than acylated counterpart. Three experiments were carried out with 4 replicas for each experiment. Values= Means ± Standard error (n=3)

CONCLUSIONS

- All anthocyanin-rich extracts inhibited colon cancer cell proliferation at varying degrees and their GI50 values are low enough to exert chemoprotective effect possibly in GI tract.
- Anthocyanins and other phenols showed additive interaction and anthocyanins in AREs play an important role on inhibition of cancer cell proliferation
- Non-acylated anthocyanins are more effective chemoprotective agents than their corresponding anthocyanins acylated with an aliphatic acid

OUTGOING RESEARCH

- To statistically analyze the effect of anthocyanidin, glycosylation, and acylation of anthocyanin on their antiproliferation of HT-29 cells
- To determine experimentally if cinnamic acid acylation in anthocyanins will increase their biological activities
- To determine experimentally if di- and tri-glycoside of anthocyanidins may reduce chemoprotective effect.
- To determine experimentally if the type of aglycones has an effect on their chemopreventive activities and cyanidin may have greater biological activities than pelargonidin.

References:

1. Giusti, M.M. and Wrolstad, R.E. *Biochemical Engineering Journal* 14, 217–225, 2003.
2. Kühnau, J., The flavonoids. *World Rev. Nutr. Diet.* 24 (117-191), 1976.
3. Hertog, M.G., et al. *Nutr Cancer* 20 (1), 21-9, 1993.
4. Glade, M.J. *Nutrition* 15 (6), 523-6, 1999.
5. American-cancer-society. *Cancer facts and figures*, 2006.
6. Hagiwara, A., et al. *Cancer Letters* 171 (1), 17-25., 2001.
7. Harris, G.K., et al. *Nutr Cancer* 40 (2), 125-33, 2001.
8. Katsube, N., et al. *J Agric Food Chem* 51 (1), 68-75, 2003.
9. Lazze, M.C., et al. *Carcinogenesis* 25 (8), 1427-33, 2004.
10. Malik, M., et al. *Nutr Cancer* 46 (2), 186-96, 2003.
11. Seeram, N.P., et al. *J Agric Food Chem* 52 (9), 2512-7, 2004.
12. Yi, W., et al. *J Agric Food Chem* 53 (18), 7320-9, 2005.
13. Zhao, C., et al. *J Agric Food Chem* 52 (20), 6122-8, 2004.
14. Andersen, Ø.M. and Jordheim, M., *Flavonoids: Chemistry, Biochemistry and Applications*, Andersen, O.M. and Markham, K. R., 2004.
15. Olsson, M.E., et al. *J Agric Food Chem* 52 (24), 7264-7271, 2004.
16. Giusti, M.M. and Wrolstad, R.E., *Journal of Food Science* 61 (2), 322-26, 1996.
17. Skehan, P.,et al. *J Natl Cancer Inst* 82 (13), 1107-12, 1990.
18. Giusti, M. M. and Wrolstad, R.E., *Current Protocols in Food Analytical Chemistry*, 1 ed., Wrolstad, R.E., et al, 2001, pp. F1.2.1-F1.2.13.
19. Waterhouse, A.L., *Current Protocols in Food Analytical Chemistry*, 1 ed., Wrolstad, R. E., et al., 2001, pp. I1.1.1-I1.1.8.
20. Durst, R.W. and Wrolstad, R. E., *Current Protocols in Food Analytical Chemistry*, 1 ed., Wrolstad, R.E., et al, 2001, pp. F1.3.1-F1.3.13.
21. He, J., et al, *J Agric Food Chem*, 53, 2859-66, 2005